Summer School
on

"Marine Fishery Resources Assessment and Management"
Central Marine Fisheries Research Institute, Cochin
May 24- June 22, 1999

REPRODUCTION IN FISHES

Dr. K. K. Joshi
Central Marine Fisheries Research Institute, Cochin

Studies on reproduction in fishes require the knowledge on the nature of gonad development. The methods employed are histology, measurement of oocytes, staging based on the appearance of whole oocytes, gonad indices and visual staging. The histological methods are reliable, but time consuming while the visual staging based on external appearance of the gonad is less reliable but fast and suitable for routine studies.

I. HISTOLOGICAL STAGING

Ovaries are classified by the presence of most advanced oocytes in the ovary, without considering the proportion of the oocytes of different stages. However, authors like Young *et al*. (1987) based their staging using the relative proportion of different types of oocytes. Recently, quantitative methods of analyzing histological sections of gonads, based on "point count" technique have been introduced.

Different stages of oocyte growth based on the conventional histological processing and staging with Haematoxylin and Eosin are as follows.

1. Chromatin nucleolar stage
2. Perinucleolar stage
3. Yolk vesicle formation
4. Vitellogenic stage (yolk)
5. Ripe stage

1. Chromatin nucleolar stage

Teleost oocytes initially arise within the ovarian luminar epithelium. Oocytes are surrounded by follicles resulting in a complex bud. Oocytes having conspicuous nucleolus are associated with chromatin threads. During the oogenesis, oogonia undergo proliferation by mitotic division and become the primary oocytes, when the chromosomes become arrested at the diplotene stage of the first meiotic prophase. In oogonia the nucleus – cytoplasm ratio is high but as growth progresses this ratio decreases. Oocytes enter a period of growth that varies from species to species, mainly by accumulation of yolk. The oocytes in this stage are usually transparent with the nucleus visible at the centre.

2. Perinucleolar stage

Nucleoli get multiplied and are arranged along the periphery of the nucleus; this stage is called perinucleolar stage. Concomitant with oocyte growth, the nucleus increases in size and multiple nucleoli appear, generally at its periphery. The late perinucleolus stage can be distinguished from the previous stage by the enlargement of the oocyte. During this period (Diplotene stage of meiosis), lampbrush chromosomes are formed which disappear immediately prior to the break down of the germinal vesicle (GVBD) during oocyte maturation. Results of the Electron microscope studies showed that the yolk nucleus is composed of various cellular organelles such as mitochondria, golgi bodies, smooth endoplasmic reticulam and lipid granules. Yolk nucleus functions as a centre for the formation of organelles with in the oocyte. At the end of the perinucleoular stage, the oocyte surface is extended into numerous microvilli, around which the chorion precursor begins to accumulate in patches. Prolonged growth phase is dependent on pituitary gonadotropins. Growth is mainly by accumulation of yolk.

3. Yolk vesicle formation

Yolk vesicle first appears in the cytoplasm during the secondary growth of oocytes. EM studies showed the involvement of endoplasmic reticulum and golgi bodies in the formation of yolk vesicle. Results suggest that the yolk vesicles are synthesized within the oocyte (auto synthetic). The stage II oocyte when seen through the low power microscope translucent and the region between yolk and outer membrane is transparent. As oocyte grows, the yolk vesicle increases both in size and number and at maturity they move to the periphery of the oocyte, when they are known as cortical alveoli. As vitellogenesis proceeds, most of the cytoplasm of mature eggs becomes occupied by yolk globules surrounded by limiting membrane.

Yolk vesicles can be stained by H & E and the cytoplasm will appear empty but this stain with Alcian blue, toluidine blue and PAS (Periodic acid-Schiff). The yolk vesicle increase in size and number to form several peripheral rows and gives rise to cortical alveoli, which release their content into perivitelline space inside the egg membrane during fertilization. They are not yolk in a strict sense since they do not serve as nutrient source for the embryo. Selman and Wallace (1989) recommended that it may be replaced by the term ‘cortical alveoli’ in future studies. But in some marine species oil droplets (fat vesicle, vacuoles or globules, fatty or lipid droplets) begin to accumulate during this period. They are involved in the formation of oil or lipid globule in fully developed eggs. The contents of these lipid droplets are dissolved during dehydration with alcohol and appear empty in conventional staining. The oocytes appear enlarged and a clear transparent space can be seen between outer membrane and yolk laden cytoplasm.

4. Vitellogenic stage (yolk)

Appearance of different types of yolk bodies in sequence is the characteristic feature of vitellogenesis. Three types of Yolk material are present in the oocytes like oil droplet, yolk vesicles and yolk globule. The appearance of yolk proteins in fluid filled sphere (yolk sphere, granule or globules) is characteristic of this stage. The granules are very small, appear 2 μ m, 3 μ m and may be difficult to detect with light microscope. The yolk spheres may maintains their integrity through oocyte growth or fuse eventually to form a continuous mass of fluid yolk which gives the eggs their characteristic transparency.

In teleost's it has been demonstrated that a female-specific protein (Vitellogenin) which is synthesized in the liver in response to 17/3-estradiol is released to blood and then transported to the ovary. Protein yolk precursors are incorporated into the oocyte by micro pinocytosis. Most yolk proteins appear to be synthesized outside the oocyte (heterosynthetic). Also an intraovarian origin of yolk proteins (auto synthetic) occurs in teleosts.

5. Ripe stage

Oocyte maturation involves the resumption of meiosis. It is commonly regarded that chromosomal activity proceeds to metaphase of the second meiotic division. After the oocyte completes its growth, it resumes the reduction division. The fully-grown oocyte posses a large nucleus (Germinal Vesicle) in meiotic prophase. The germinal vesicle cannot be seen by external observation because of the opaque cytoplasm. Maturation is completed by the peripheral migration and dissolution of the germinal vesicle (GVBD). GVBD is the indicator of oocyte maturation.

The first visible event associated with final oocyte maturation is the migration of germinal vesicle to the animal pole, where the micro pile is situated and at this stage, the germinal vesicle becomes visible under the dissection microscope. The membrane of germinal vesicle then breaks down (GVBD) and its contents become intermingled with the cytoplasm. These include the coalescence of lipid droplets and yolk globules, a further rapid size increase of the oocyte size is caused by hydration and an overall increase in oocyte translucency occurs. The oocyte appears transparent and two or three oil droplets can be seen. After the completion of first meiotic division, the oocytes (now eggs) are expelled into the ovarian cavity (ovulation). The nucleus of first polar body follows before the oocyte is ovulated into the lumen. In many mature teleosts. There is further rapid increase in size due to hydration of the oocytes.

II. MEASUREMENT OF OOCYTE SIZE

Size of the oocytes is used as a measure of development by measuring oocyte diameter and classifying the ovaries according to the position of the large mode. Different approaches are followed in this, like measuring the average diameter of the most advanced group of oocytes, average maximum diameter of largest oocytes and diameter

of the largest single oocyte, and a correlation is found between the largest oocyte diameter and size of the most advanced mode and the measurement of the maximum (largest) oocyte diameter is considered to be the easiest method of establishing the stage of the fish.

The relationship between the size of ripe eggs and fish size is complicated. In general, egg size increases with the age and size of the females. Young recruit spawners give smaller eggs than older recruit and repeat spawners. Regression of egg length against length of fish was only significant if these very young recruit spawners were present in the sample. The relationship between the length of female and the diameter of their eggs was significant from smaller size of eggs from second and third year old fish. But in older fish 4-8 years, there was no relationship between body length and egg size. In small, short lived fish, the narrow range of sizes and ag4es for spawning adults is the reason for lack of a significant relationship between female size and egg size. Oocytes particularly small unyolked ones are rarely perfectly spherical in shape and, three approaches are there to measure them.

1. ***Single measurement of oocyte diameter on a random orientation basis. (conventional methods)***
2. ***An average measurement based on measurement of the minimum and maximum diameters.***
3. ***The longest dimensions in species with elongate oocyte.***
4.

III. STAGING BASED ON THE MICROSCOPIC APPEARANCE OF WHOLE OOCYTES

Oocytes fall into three categories: unyolked oocytes are transparent, yolked ones are opaque and ripe ones are transparent. There is semi-opaque category between the transparent unyolked oocytes and the opaque yolked oocytes. An examination of the ovary stage of development shows the following categories of ova :- 1) immature ova: minute transparent ova processing a nucleus and proto Plasmic layer. 2) Maturing ova: small, opaque ova, yolk formation started, but not completed. 3) Mature ova : opaque ova, yolked in the follicle 4) large fully or partially transparent ova released from the follicles.

HISTOLOGICAL METHODS

1. TESTIS

Dissect the fish and collect the fresh milt. Put it on a dry glass slide. Add Marine-Fish-Ringer solution. Smears of milt are prepared on glass slide. Then dry the slides in air and stain with 2% acetoorcein for 3 minutes. Wash the stained slides in running water.

Histology

1. Cut the tissues into small pieces
2. Fix in 10% Neutral Buffered Formalin (other fixatives like Bouins and Zenker can be used)3
3. Wash the tissue in running tap water after 24 hours. (If Bouins is used was until the yellow colour of the Bouins fluid is removed).
4. Dehydrate in a series of alcohols from 30% to 100% (for storage purpose tissue can kept in 70% ethyl alcohol)
5. Clear the tissue in chloroform or xylene.
6. Transfer the transparent tissue into the molten wax (melting point 52-54 C).
7. Make blocks after the infiltration is completed.
8. Cut sections at 5-7 / u m thickness.
9. Apply a drop of Mayer's glycerol albumen to the glass slides
10. Spread the sections over the slides with the help of hot plate.
11. Deparaffinise the sections in xylene.
12. Hydration of slides in series of alcohols from 100% to 30%.
13. HEIDEN HAN'S STAIN …. 30 SECONDS – 2 MINUTES. Or HARRIS HEMATOXYLIN
14. Rinse in running tap water to remove excess stain and place in moderately warm water to "blue" sections.
15. EOSINE (1% aqueous) …… 2 to 3 minutes
16. Dehydration 30% alcohol …….. 2 minutes
    50% alcohol …….. 2 minutes
    70% alcohol …….. 2 minutes
    95% alcohol …….. 2 minutes
    Absolute alcohol …….. 2 changes
17. Alcohol-xylene (If sections look milky, dehydration is not complete. Take back to absolute alcohol)
18. Xylene …….. Until sections are clear.
19. Mount in DPX

2. OVARY

Bouins fixative is used mainly for the first two stages of ovaries, whereas Smiths Dichromate is recommended for later vitellogenic ovaries in which yolk is enormous. Single embedding in wax will create problems while cutting the sections. To avoid extreme shrinking and wrinkling of oocytes and collapse of Zona radiata, celloidin coating or double embedding with celloidin is recommended.

1. Cut the ovary into small pieces.
2. Fix in NBF/BOUINS or SMITHS DICHROMATE.
3. After 24 hours of fixation, wash the ovarian tissue in running tap water.
4. The tissue is then dehydrated in a series of alcohols from 30% to 100%. (For storage purpose keep in 70% alcohol)
5. Clear in chloroform

6. Single embedding in wax for the first two stages of ovary. Celloidin coating or double embedding for advanced stages of vitellogenic ovary.
7. Cut sections at 5-7 /u m thickness. Cut surface of the block should be given painting with a supporting agent. This will provide additional support to the yolk. The supporting agent is a 2% celloidin in 50% ethanol-ether mixture. Paint this agent on the block surface prior to cutting each section. Another method to avoid problem of extreme shrinking and collapse of zona radiate is double embedding.

DOUBLE EMBEDDING

a) Take paraffin embedded tissue.
b) Melt paraffin from the tissue in oven.
c) Clear in toluene 1-2 hours (2-3 changes)
d) Absolute alcohol three changes (30 minute – 1 hr.)
e) Place in 1% Celloidin in Methyl Benzoate to which an equal amount of alcohol is added. Place tissue gently on top of the solution. Tissue will gradually sink in celloidin (12-24 hrs)
f) Transfer tissue to 3% Methyl Benzoate Celloidin for 48-96 hrs.
g) Place tissue directly into dehydration and leave overnight
h) Infiltrate in 58-60 C in paraplast. 3 to 4 changes (Leave in oven overnight)
i) Embed in paraplast and cut. (Steps d, e & f should be carried out in hood)

8. Apply a drop of Mayer's glycerol albumen to the glass slide.
9. Spread the tissue sections over the slides with the help of hot plate.
10. Deparaffinise the sections in Chloroform/Xylene
11. Hydration of slides in a series of alcohol from 100% to 30%
12. Stain in EHRLICH'S HEMATOXYLIN or
    HARRIS HEMATOXYLIN or
    HEIDEN HANS HEMATOXYLIN
13. Rinse in running tap water
14. EOSINE (1% Aqueous)/Mallory stain
15. Dehydration through 30%-95%, absolute alcohol
16. Alcohol-Xylene
17. Xylene
18. Mount in DPX

FIXATIVES

I. BOUIN'S

Saturated Picric Acid ----- 75 ml
37-40% Formaldehyde ---- 25 ml
Acetic acid ---------- 5 ml
Time : 4 to 18 hours
Wash in running water, several
Changes of 70% alcohol

II. NEUTRAL BUFFERED FORMALIN

37-40% Formaldehyde ---- 25 ml
Water ---------- 100 ml
Sodium phosphate
Monobasic ---------- 4 gm
Sodium phosphate
dibasic ---------- 4 gm

III. ZENKER

Mercuric chloride ---- 50 gm
Potassium dichromate ---------- 25 gm
Sodium sulfate ---------- 10 gm
Distilled water ---------- 1000 ml
Time : 6 to 24 hours

IV. SMITH'S DICHROMATE

5% Potassium Dichromate ----- 8.75 ml
37-40% Formaldehyde ---- 10.0 ml
Acetic acid ---------- 2.5 ml

MAYERS GLYCEROL

1 Glycerol : 1 Fresh egg white

STAINS

I. HARRIS HEMATOXYLIN

Hematoxylin crystals / --------- 50 gm
Powder
Absolute alcohol ---------- 50 ml
Potassium/Aluminium
Sulfate ---------- 100 gm
Distilled water ---------- 1000 ml
Mercuric oxide (red) ---------- 2.5 gm

Dissolve hematoxylin in alcohol, and the alum in water- by aid of heat. Then mix two solutions. Bring rapidly to boil. Remove from heat, add mercuric oxide slowly.

Reheat until it is dark purple. Remove from heat and place vessel in a pan of cold water until it cools. Before use add 2 ml Acetic acid to 100 ml stain. Filter.

II. EHRLICH'S HEMATOXYLIN

Hematoxylin ---------- 2 gm
Glycerine ---------- 100 ml
Distilled water ---------- 1000 ml
Glacial acetic acid ---------- 100 ml
Potash alum ---------- 10-14 gm

Dissolve hematoxylin in the alcohol before adding other ingredients. The stain may be ripened naturally by allowing to stand in a flask, loosely stoppered with cotton wool, in a warm place and exposed to sunlight. The flask should be shaken frequently and ripening takes some weeks. When good staining is attained on the test slide, the solution is bottled. Filter before use. The Hematoxylin may be partially oxidized and the stain used immediately, by the addition of 0.3 g Sodium iodate to the above.

III. HEIDENHANS IRON HEMATOXYLIN

Mordant and differentiation

Ferric ammonium sulfate ----- 5 gm
Distilled water ---------- 100 ml

Use only the clear, violet crystals of alum, not those that have become opaque and yellowish green. Dissolve without heat.

Hematoxylin stain

Hematoxylin ---------- 0.5 gm
Absolute alcohol ---------- 10 ml
Distilled water ---------- 90 ml

Dissolve the Hematoxylin in the alcohol and then add the water. Allow to ripen for a few days and store in a tightly stoppered bottle.

REFERENCES

Nagahama, Y. (1983). The functional morphology of teleost gonads. In fish physiology Vol. IX : Reproduction. (Eds. W.S. Hoar, D.J. Randall and E.M. Donaldson) pp. 223-75 (Academic Press: New York)

Young, J. W., Blaber, S. J. M., and Rose, R (1987). Reproductive biology of the three species of midwater fishes associated with the continental slope of eastern Tasmania, Australia. *Marine Biology* (Berlin) 95: 323-329.

West, G (1990). Methods of assessing ovarian development in fishes, a review. *Aust. J. mar. Freshwater Res.* (41) : 199-222.

Wallace, R. A., and Selman, K. (1979). Physiological aspects of oogenesis in two species of stickle backs, *Gasterosteuns aculeatus* L. and *Apettes quadracus* (Mitchill). *Journal of fish biology* 14:551-564

****